# Diversity Analysis of Begomoviruses Associated beta Satellite Molecules

**A**
**RESEARCH PLAN**
**SUBMITTED**

**FOR THE PARTIAL FULFILLMENT OF**

**Doctor of Philosophy (Ph.D.)**


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**Introduction:-**

Geminiviruses consist geminate shaped virions which is about 18-30nm in size. The family geminiviridae is currently divided in seven genera, Becurtovirus, Begomovirus, Curtovirus, Eragrovirus, Mastrevirus, Topocuvirus and Turncurtovirus based on their genome organization, insect vector and host range (Brown et al,2012;Adams et al, 2013) . These viruses are characterized by single-stranded circular DNA (ssDNA) genome and replicate in the nuclei of the host cells. The genus Begomovirus, with its more than 200 species, is the most represented. It includes viruses with either monopartite or bipartite genomes, transmitted by the whitefly *Bemisia tabaci* Genn., infecting a wide range of dicotyledonous plants in both the old world and new world.

In a developing country like India, majority of the population is still dependent entirely upon the farming for their livelihoods. Crops can be affected by various diseases caused by biotic or abiotic factors exhibiting a wide range of symptoms. Among the biotic factors, diseases caused by plant viruses in extreme cases can reduce the yields to zero, leading harmful effect on mankind. Begomoviruses are responsible for major crop loss in India which is the foremost reason for strong research interest these viruses have created. Begomoviruses have been reported from at least 16 different groups of crop in India (Dasgupta*et al.*, 2012). In the last three decades, insects belonging to the genus *Bemisia* have become one of the major pests in the worldwide agricultural systems (Naranjo and Ellsworth, 2001; Jones, 2003). *B. tabaci* is very prevalent in hot and humid climate, in such conditions dual affect of virus infection and vector feeding/multiplication is highly damaging to the crops. Hence, due to presence of begomoviruses and their vector in ample quantity, these infections/diseases are a serious constraint to food, fiber and ornamental plants.

The majority of OW monopartite begomoviruses are associated with additional ssDNA molecules. The first evidence for this came with the report by Dry et al. of an ssDNAs satellite associated with tomato leaf curl virus (ToLCV) occurring in Australia. This molecule was later shown to be a defective(truncated) version of a much larger group of subviral components associated with begomoviruses that are now known collectively as beta satellites (Briddon et al.,2003). Beta satellites are approximately half the size of a begomovirus component (~1,360nts) and are required by the helper begomovirus for efficient infection of some hosts (Briddon et al.,2006, Xie et al.,2010, Patil et al.,2010).Betasatellites have been shown to be

associated with an increasing number of diseases caused by begomoviruses, including many of the most significant, economically damaging diseases occurring in the OW.(Nawaj-ul-rahman, 2010)

The betasatellites are very diverse in nature and are associated with helper begomoviruses. Betasatellites contain a region (~100 nt) known as the satellite conserved region (SCR), which is

highly conserved between all betasatellites and contains the stem loop. In addition, the betasatellites contain an adenine rich region (A-rich) and a single ORF (the *βC1* gene) in the complementary orientation of the molecule.

The precise function of betasatellites and its βC1 protein in pathogenesis of viruses although it has been proposed that DNA *β* may play a direct or an indirect role in replication, facilitating movement, or countering host defense response.

Begomovirus infected plants often exhibit yellowing of leaf veins, yellow mosaic patterns, curling of leaves, leaf distortion, decrease in leaf size and stunting in plants.

**Aims of the study:**

The main focus of this thesis is to reveal the diversity of complexes with monopartite begomoviruses and DNA-satellites infecting crops, ornamental plants and weeds by analyzing the components of two complexes from North India. The prevalence and genetic diversity of begomoviruses in these areas will be discussed. Moreover, the interactions between begomoviruses and satellites from different regions are also going to address, which will enhance our knowledge about the adaptability and evolution of new begomovirus complexes.

The specific objectives are:

1. Identification of the begomoviruses and their possible alternative hosts in Northern India
2. Molecular and biological characterization of begomoviruses and associated betasatellites.
3. Diversity and recombination analysis of beta satellite molecules associated with different begomoviruses.

4. Co-inoculation of begomoviruses and associated beta satellite molecules to assess the compatibility and trans-replication capability of begomoviruses and satellites from different geographic regions

**Review of Literature:-**

Viruses are "Intracellular parasites with nucleic acid capable of directing their own replication that do not serve any essential function for their host, have an extra-chromosomal phase and are not cells" (Roossinck, 2011) In plants, viruses are casual agents of ~47% of all emerging diseases (Anderson *et al.*, 2004). Geminiviruses belong to the family *Geminiviridae*, which is the largest plant virus family. Begomoviruses are the largest group from seven genera of family *geminivirdeae.* They infect a broad range of plants including crops, ornamental and weed plants all over the world.

Begomoviruses are devided into two groups Old World and New World viruses according their origion. All begomoviruses native to the New World (NW) and a small number originating from the Old World (OW) have bipartite genomes (with components known as DNA-A and DNA-B). The majority of the OW begomoviruses have genomes consisting of a single component homologous to the DNA-A component of the bipartite viruses. Begomoviruses from the NW and OW are genetically distinct. They segregate separately in phylogenetic analyses, and the OW viruses show a greater genetic diversity and have an additional, absolutely conserved gene (known as V2 for the monopartite and AV2 for the bipartite viruses) that is absent in the NW begomoviruses.

The genomes of monopartite (and DNA-A components of bipartite) begomoviruses are typically ⍰2.8 kb in length and have genes in both orientations from a non-coding intergenic region (IR), which contains promoter elements and the *ori* of virion-strand DNA replication. The virion strand *ori* consists of a predicted hairpin structure containing a conserved (between geminiviruses) nonanucleotide (TAATATTAC) sequence in the loop and repeated upstream motifs known as "iterons". The DNA-A component of begomoviruses contains either five or six ORFs in both orientations that encode ~10 kDa proteins. The virion-sense strand of most

begomoviruses encodes two proteins coat protein (CP) and pre coat protein (V2).Coat protein required for encapsidation, insect transmission and movement in plants (Sharma & Ikegami, 2009) while pre coat protein is a pathogenicity determinant and act as a suppressor of RNA silencing (Yadava *et al.*, 2010). The complementary-sense strand encodes four proteins, C1, C2, C3 and C4. Replication associated protein (C1) is the only virus-encoded product required for viral DNA replication. C2 (Transcriptional activator protein TrAP) Up-regulates the late (virion sense) genes (for bipartite begomoviruses), serves as a suppressor of RNAsilencing in bipartite (Yang *et al.*, 2007; Trinks*et al.*, 2005) and monopartite begomoviruses (Gopal*et al.*, 2007).Replication enhancer protein (REn; C3) is involved in establishing an environment conducive for virus replication (Pedersen & Hanley-Bowdoin,1994). The function of the C4 protein still remains unclear but for some viruses it is a pathogenicity determinant and a suppressor of PTGS by binding of siRNAs (Pandey*et al.*, 2009; Vanithara*et al.*, 2004)

In bipartite viruses DNA B component encodes one gene, BV1 (the nuclear shuttle protein [NSP]) in the virion-sense and one gene, BC1 (the movement protein [MP]), in the complementary-sense. These gene products have been implicated in local and systemic spread, symptom development, host range determination and virus transmission by whiteflies. The alpha satellite associated with CLCuD is approximately half the size of its helper begomovirus (1370 nucleotides) and encodes a Rep. It can replicate autonomously in the cells of host plants but depends on the helper begomovirus for encapsidation and movement both within and between plants. Although the discovery of alphasatellites did not resolve the etiology of either disease, because presence of respective alphasatellites did not affect the symptoms of the disease (Mansoor*et al.,* 1999; Saunders and Stanley, 1999) it did stimulate the search for additional components associated with these diseases.A

Betasatellites depend entirely upon their helper begomoviruses for their replication, movement in plants, and transmission between plants, apparently by trans-encapsidation in the helper virus coat protein (CP).

All functions of beta satellites described are mediated by the βC1protein. The protein has multiple functions: pathogenicity determinant(determines the symptoms of the infection), suppressor of post transcriptional gene silencing (PTGS) to overcome host plant defence,

potential involvement in virus movement *in planta* (Saeed*et al.*, 2007), up regulation of viral DNA levels *in* plant (Briddon*et al.*, 2001; Saunders *et al.*, 2000), potential binding of DNA/RNA (Cui *et al.*, 2005) as well as interaction with a variety of host factors (Eini*et al.*, 2009) and the CP of helper virus(Kumar *et al.*, 2006). Moreover, βC1 protein encoded by betasatellites also form multimeric complexes, which are essential for symptom induction ininfected plants (Cheng *et al.*, 2011).

**Methodology:-**

**1.Collection of plant samples:**

The infected leaf samples will be photographed with a high resolution camera from the different regions of North India on the basis of typical begomovirus symptoms i.e., yellowing of leaf veins, yellow mosaics patterns, curling of fractional or complete leaves, leaf distortion, chlorosis and decrease in leaf size and stunting in plants. Young leaves of plants will be collected, kept in plastic bags labeled with permanent marker, carried on dry ice or liquid nitrogen and stored at -20˚C until utilized.

**2.Genomic DNA extraction:**

Total DNA from leaf samples will be extracted by using a CTAB (Cetyl Tri-methyl Ammonium Bromide) method (Saghai-Maroof*et al.*, 1984). The DNA pellet will be washed with cold 70% ethanol twice, vacuum dried, and resuspended in 100 µl of TE buffer.

**3.Estimation of DNA quantity and quality:**

Concentration of DNA will be estimated through NanoDrop (Cuvette free spectrophotometer) to check the quantity of DNA/µl for further study. DNA quality will also check by running the DNA samples on 0.6 % agarose gel using 1X TAE. The gel will stained with ethidium bromide (0.3 mg per ml) and read under UV light on a transilluminater or Gel-doc system.

**4.Screening byPCR:**

For amplification of DNA by PCR a reaction mixture of 50µl containing 10pg- 1µg template DNA, 5µl 10X *Taq* polymerase buffer, 5µl 2mM dNTPs, 1.5mM MgCl2, 0.5µM of each primer

and 1.25 units *Taq* DNA polymerase will prepared in a 0.25ml thin walled PCR tube. The reaction mixture will carry out in thermal cycler,using begomovirus specific universal primers against coat protein (CP) and β-satellite. This will be confirming the molecular level infection of the begomovirus in plant samples.

**5.Rolling Circle Amplification (RCA) and Restriction digestion :**Full length sequences of DNA-A and betasatellite will be obtained by rolling circle amplification of cp positive samples ,using Templiphi kit (GE Healthcare) followed by restriction digestion with different restriction enzymes.

**6.Sequencing and Submission of sequences in NCBI and Homology search**

Sequencing of the amplified product will be carried out by using after sequencing the DNA sequence will be submitted to NCBI-Bank It or Sequin. Homology search will be carryout by using alignment tools (e.g., NCBI-BLASTn etc.).

**7. Phylogenetic study and Recombination analysis:**

Database search will be carried out with BLAST*n* or with other BLAST tools(http://www.ncbi.nlm.nih.gov/BLAST). MEGA version 5.2 (http://www.megasoftware.net) will be used for Pair wise and Multiple sequence alignments through its CLUSTAL- W program and Phylogenetic trees will be constructed by using Neighbor-Joining algorithm. Recombination analysis will be carried out using RDP4 programme

**8.Construction of clones:** Full length DNA-A and beta satellites will be cloned in vectors like pUC19or pGMT vectors. The resulted amplicon will cloned in the binary vector pCAMBIA 1301 from pUC19.

**9.Agroinfiltration:**

A grown culture of *A. tumefaciens*LBA4404strain harboring the different pCAMBIA1301 a will be used for agroinfiltration on abaxial side of host plant leaves and transformed leave of *N. benthamiana*plants, incubate in growth chamber at 25°**C** for 48-76 hrs

**10. Screening of Transformed plants using PCR:**

Total DNA will extract from young leaves of transgenic plants by CTAB method (Saghai-Maroof*et al.*, 1984). To confirm the transgene, PCR will carry out with specific primers. PCR products will monitored by 1.2 % agarose gel electrophoresis and ethidium-bromide staining.

**11.Southern blot analysis of transformed plants:**

Genomic DNA will be digested with restriction enzymes and separated by restriction enzymes according to the size of DNA fragments. Afterthen DNA will be transferred on nitrocellulose membrane by capillary flow method. Hybridization between target DNA and labeled probe will be visualized on X-ray film or by development of colour will confirm the presence and copy number of transgene.

**12. Publication of research results and thesis writing.**

Publication of research results in National and International journals, books, symposiums and conferences.

**Significance of work:-**

1. Northern India climatic conditions support the high population of *Bemisiatabaci* (whitefly). This study will give the information about the natural host plants of begomoviruses and beta satellites in Northern states.
2. Recombination analysis will help in revealing possible emergence of new begomovirus complexes.
3. Co-inoculation of begomoviruses and different beta satellites will help in knowing the role of beta satellites in disease symptom induction and exchange of beta satellites among different geographical regions occurring begomoviruses.

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